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Ethyl methanesulfonate toxicity in Viracept—A comprehensive human risk assessment based on threshold data for genotoxicity

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ABSTRACT

Based on a production accident Viracept (nelfinavir mesilate) tablets, an HIV protease inhibitor supplied by Roche outside the US, Canada and Japan was contaminated with relatively high levels of ethyl methanesulfonate (EMS) for at most 3 months in spring of 2007. On the basis of a wide variety of toxicological data including critical experiments for mutation induction under chronic exposure conditions and cross-species exposure scaling experiments to extrapolate to humans, we estimate the added risk of adverse effects (cancer, birth abnormalities, heritable defects) in any individual patient accidentally exposed to EMS via contaminated Viracept tablets in the context of this production accident as essentially zero.

Of critical important for this risk assessment are pivotal *in vivo* genotoxicity studies (MNT, MutaMouse) providing evidence for 'hockey-stick', like dose–response relationships for the risk defining induction of gene mutations and chromosomal damage by EMS [Gocke, E., Müller, L., Pfister, T., Buergin, H., 2009a. Literature review on the genotoxicity, reproductive toxicity, and carcinogenicity of ethyl methanesulfonate. Toxicol. Lett.; Gocke, E., Müller, L., Pfister, T., 2009b. EMS in Viracept—initial ('traditional') assessment of risk to patients based on linear dose response relations. Toxicol. Lett.; Gocke, E., Müller, L., Ballantyne, M., Whitwell, J., Müller, L., 2009c. MNT and MutaMouse studies to definde the in vivo dose-response relations of the genotoxicity of EMS and ENU. Toxicol. Lett.]. As outlined in Gocke and Wall [Gocke, E., Wall, M., 2009. In vivo genotoxicity of EMS: Statistical assessment of the dose response curves. Toxicol. Lett.], several statistical approaches are in support of a threshold model to best fit the data. The presence of clear no effect levels in bone marrow, liver and GI-tract tissue with several dose levels tested below the NOEL permits the calculation of safety factors with considerable confidence. In calculating the ratio of the NOEL dose in the animal studies (25 mg/kg/day) divided by the calculated maximal daily dose of the patients (1068 ppm EMS in 2.92 g Viracept tablets = 2.75 mg EMS or 0.055 mg/kg for a 50 kg person) we derive a safety factor of 454 based on oral intake. Detailed absorption, distribution and metabolism studies in mice, rats and monkeys and with human surrogates *in vitro* enable us to estimate the safety factors also for the calculated likely highest exposure (AUC and C_{max}) of patients to EMS [Lave, T., Birnböck, H., Götschi, A., Ramp, T., Pähler, A., 2009a. *In vivo* and *in vitro* characterization of ethyl methanesulfonate pharmacokinetics in animals and in human. Toxicol. Lett.; Lave, T., Paehler, A., Grimm, H.P., 2009b. Modelling of patient EMS exposure: translating pharmacokinetics of EMS in vitro and in animals into patients. Toxicol. Lett.]. We calculate the total exposure (AUC) based safety factor to amount to at least 28. This lower value is due to the conservative prediction of a longer half-life of EMS in man versus mouse, rat and monkey. Based on the estimated human C_{max} the safety factor for affected Viracept patients is calculated to be 370, as C_{max} is mainly dependent on volume of distribution, which is not much different for EMS in different species. We consider that the total exposure based safety factor constitutes a minimal value since the considerations regarding evidence of error-free repair at sub-threshold concentrations argues in favor of using the highest EMS concentration (C_{max}) rather than the AUC as basis for risk assessment. The 'true value' very likely lies somewhere between these two numbers as aspects such as repair enzyme availability and status of the cell cycle relative to the insult are important parameters that may not fully support safety factors based solely on C_{max} estimates.

Potential adverse effects of EMS such as cancer, birth abnormalities and heritable effects are considered to be sequelae of its genotoxic activity. Hence, the thresholded dose–response relationships should also

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apply to these endpoints. We also provide a comprehensive discussion of the specific disease situation of the HIV infected target population and potential influences of co-medications on the susceptibilities and repair capacities of EMS induced DNA lesions.

1. Methods to calculate risk for "EMS in Viracept"

The accidental contamination of Viracept (nelfinavir mesilate) tablets, an HIV protease inhibitor supplied by Roche outside the US, Canada and Japan with relatively high levels of EMS for at most 3 months in spring of 2007 triggered an array of activities to judge the risk of patients who have taken these tablets. The reasons for this contamination and the course of events are published in other contributions to this issue of Toxicology Letters (Gerber and Toelle, 2009; Müller and Singer, 2009). In our risk assessment as provided in the following, we were guided by the expectation that an array of non-clinical experiments *in vivo* would enable us to conduct a better quantitative risk assessment for carcinogenicity and teratogenicity for the putatively affected patients than was possible on the available data at the time of the accident (Gocke et al., 2009a,b,c). These experiments were designed in agreement with regulatory authorities as described in Müller and Singer (2009). As reviewed in Gocke et al. (2009a), numerous *in vitro* and *in vivo* studies give clear evidence for the genotoxic activity of EMS *in vitro* and *in vivo*. EMS reacts readily with DNA leading to alkylation (specifically ethylation) of nucleotides at various sites. Based on the direct DNA damaging property of EMS the dose–response relationship has been thought to be largely linear ('one hit one target' theory).

Only recently the notion that sublinear, even thresholded, dose–response relations may exist at low dose levels was shown experimentally *in vitro*. At low exposure the cellular defensive mechanisms, such as DNA repair, have been shown to be capable of effectively removing the genotoxic lesions induced by EMS in human lymphoblastoid cells *in vitro* (Doak et al., 2007). This protection led to consider the presence of 'pragmatic' threshold (or 'no observed effect levels', NOEL) regarding induction of gene mutations as well as regarding induction of chromosomal aberrations. *In vivo*, data presented by Jansen et al. (1995) for HPRT mutations in T-lymphocytes in the rat were supportive of a non-linear dose effect relationship for EMS after a single dose. Toxicity data on EMS other than data to judge genotoxicity, carcinogenicity and teratogenicity did not exist in the published literature.

The following methods were used by us to investigate the toxicity of EMS and to extend the limited genotoxicity data to the *in vivo* level with repeat dose experiments and to establish a comprehensive risk assessment for the potentially affected patients in the "EMS in Viracept" case:

(1) An *in vivo* 4-week repeat dose toxicity study in the rat with a subsequent recovery phase to establish data about the organ toxicity of EMS (Pfister and Eichinger-Chapelon, 2009).
(2) *In vivo* single and repeat dose (7 and 28 days) genotoxicity studies in the mouse to investigate the full dose–response for chromosome damage and mutations in the critical tissues GI-tract, bone marrow and liver (Gocke et al., 2009c). The experiments were designed to fulfil regulatory guidelines for these studies and included several dose levels below a presumed NOEL as well as several control groups to provide a basis for a comprehensive statistical analysis.
(3) A comprehensive analysis of the data by statistical methods to investigate which dose–response model would fit the data best (Gocke and Wall, 2009).
(4) A comprehensive *in vivo* DMPK program to characterize the kinetic behaviour of EMS in mice, rats and monkeys (Lave et al., 2009a,b).
(5) A comprehensive *in vitro* DMPK program to predict human metabolism and reliably simulate the kinetic behaviour of EMS in humans (Lave et al., 2009a,b).
(6) A comprehensive simulation of likely exposure of Viracept patients to EMS (Lave et al., 2009b).
(7) A comprehensive assessment of possible confounding risk factors of relevance for the HIV infected patients having possibly taken EMS via Viracept (see Section 4).

In the design of our experiments we were aware of the fact that the confidence in a no observable mutation level (NOEL) being a practical or even real threshold grows with the range and number of doses investigated below the NOEL. Hence, special attention has been paid to testing several adequately spaced dose levels below a presumed NOEL and to support such data with an extended negative control group of animals. This paper takes the data provided in previous sections of this special issue to the level of a risk assessment using evidence for a practical threshold of *in vivo* genotoxicity of EMS to assure the medical community as well as potentially affected patients in the "EMS in Viracept" case that there is very likely no excess risk for genotoxicity, teratogenicity and carcinogenicity versus the individual background risk in any affected patient. Further, we have asked known experts in the field to critically comment on our data and to give an outlook on which kind of future activities might be considered important to reassure the scientific community of the existence of a real threshold for mutation induction *in vivo* in steady state for EMS and other alkylating agents (Walker et al., 2009).

2. Risk assessment based on thresholded dose–response observed for *in vivo* genotoxicity

EMS is a comparatively simple molecule. Its capacity to alkylate biomolecules has long been established and is well understood. As consequence of this chemical property the generation of mutations and chromosomal aberrations have been observed in a multitude of genotoxicity studies in somatic and germ line cells in rodent species (Gocke et al., 2009a). It is generally understood, that the genotoxic property of EMS is at the basis of its tumourigenic and teratogenic properties. Further, we initially assumed that EMS does not exert any critical organ toxicities of importance beyond these properties. To support this assumption we have performed a 4-week general toxicity study with EMS in rats. This study (Pfister and Eichinger-Chapelon, 2009) indicated no other toxicities than those expected from the cytotoxic, replication inhibitory activity in rapidly dividing cells of the body and related effects in haematological and lymphatic organs.

Our *in vivo* genotoxicity studies consisted of a 7 days repeat dose micronucleus test with bone marrow and single and 28 days repeat dose mutation study with analysis of bone marrow, liver and GI-tract tissue in the mutamouse test model with EMS and ethyl nitrosourea (ENU). Dose fractionation in the transgenic mutation study permitted an assessment of the cumulative mutational behaviour of both test compounds. The full results of theses studies with EMS and ENU are provided in Gocke et al. (2009c). The statistical assessment of

these genotoxicity data consisted of four steps (Gocke and Wall, 2009):

(1) Comparison of control groups (to allow cumulation).
(2) Rejection of linear dose–response relationship (entire dose range).
(3) Acceptance of linear dose–response relationship below the NOEL.
(4) Application of threshold software developed by Lutz and Lutz (in press) to calculate the threshold values incl. confidence limits.

The effects seen in both, the vivo micronucleus test with bone marrow as well as the 28 days *in vivo* transgenic mutation study with cells of liver, bone marrow and the GI tract, best fit a hockey-stick statistical model. The assumption of a linear dose–effect relationship could be refuted with confidence.

On this basis tumourigenic or teratogenic effects are not expected at doses which do not induce mutagenic or chromosome damaging effects, i.e. at doses below a threshold for mutagenesis. Hence, we further argue that is it possible to calculate safety factors (SF) based on the clear no observed effect levels (NOEL) observed for EMS in our genotoxicity studies.

In the absence of reliable data on uptake, distribution, metabolism and excretion of the test compound in various species including humans, toxicological risk assessments are often based on dose (in mg/kg) and empirical safety factors are used to account for any species differences in reaction in safety. For a more reliable human risk estimation and since drug distribution, metabolism and excretion will usually vary between different species the concentration of the drug at the target, and it's time dependence, needs to be established based on actual total exposure (AUC) and/or maximal concentration (C_{max}). Thus, we have conducted experiments to measure stability and metabolism of EMS in *in vitro* experiments (blood, buffer and liver microsomal preparations of different species) and the exposure to EMS *in vivo* in rats and mice in terms of C_{max} and AUC of free EMS. We have linked these data to determination of adducts of EMS to the terminal valine of globin in mice, rats and monkeys. From these data, we have build a human C_{max} and AUC calculation for the desired dose ranges.

2.1. Dose based risk assessment for EMS in Viracept

As detailed in the genotoxicity review of EMS (Gocke et al., 2009a) we have established the following threshold dose level (Th_D) in mice:

Th_D = 25 mg/kg/day	Daily gavage of EMS for 7 days did not induce micronuclei at doses up to 80 mg/kg/day in mouse bone marrow in a micronucleus study (Gocke et al., 2009c) Daily gavage of EMS for 28 days did not induce mutagenic effects in the lacZ gene of MutaMouse™ animals at dose levels up to 25 mg/kg/day in bone marrow and GI tract, and at dose levels up to 50 mg/kg/day in the liver (Gocke et al., 2009c)

The estimate of the maximal daily dose of EMS in the Viracept patients is

D_{Hmax} = (2920 mg × 0.001068)/50 kg = 0.062 mg/kg/day = (2920 mg × 0.001068)/70 kg = 0.045 mg/kg/day	The maximally contaminated API batches led to the production of Viracept tablets with a contamination of about maximally 1068 ppm of EMS during treatment. The daily dose of Viracept for a patient is 2.92 g/day; spec. 10 tablets with 292 mg nelfinavir mesilate (250 mg nelfinavir). Patient weight has been used as either 50 kg or 70 kg in the literature
For further calculations we take 0.055 mg/kg/day	The 0.055 mg/kg value used throughout this paper has been estimated based on data for a contaminated batch that was likely taken for the longest time of ~3 months and is considered to represent a reasonable cautionary value. The batch contaminated with ~1068 ppm EMS was likely taken for a maximum of 1 month only

Consequently the safety factor based on a comparison of the doses amounts to

$$SF_{dose} = \frac{Th_D}{D_{H\,max}} = 25\ \text{mg/kg/day kg/0.055 mg/kg/day} = 454$$

Fig. 1 displays the genotoxic effects observed in the micronucleus test and in transgenic animals in fold changes relative to the background incidences in the respective controls.

2.2. Exposure based risk assessment for EMS in Viracept

A dose-based risk assessment is a rather limited and incomplete approach when extrapolations between species are involved. Hence, for risk assessment in the "EMS in Viracept" case, there was a need to relate the effect observed in genotoxicity studies to the internal exposure to EMS and to scale this exposure to the likely human exposure at the level of contamination of Viracept tablets. The approach to estimate EMS exposures in animals and human is summarized in the following. Details are provided in Lave et al. (2009a,b).

(1) Pharmakokinetic studies with radiolabelled EMS were conducted in animals.
(2) EMS pharmacokinetic data in animals were linked to haemoglobin adduct levels.
(3) The half-life of EMS in humans was estimated based on animal data and *in vitro* data with stability/degradation in buffer, liver extracts, human plasma and haemoglobin.
(4) The model derived from the *in vitro* data was validated using real data from mice and rats.
(5) Using data from Steps 1 through 4 we used this model to estimate patient exposure to EMS.
(6) Safety factors were calculated from data relative to general toxicity and genotoxicity animal data.

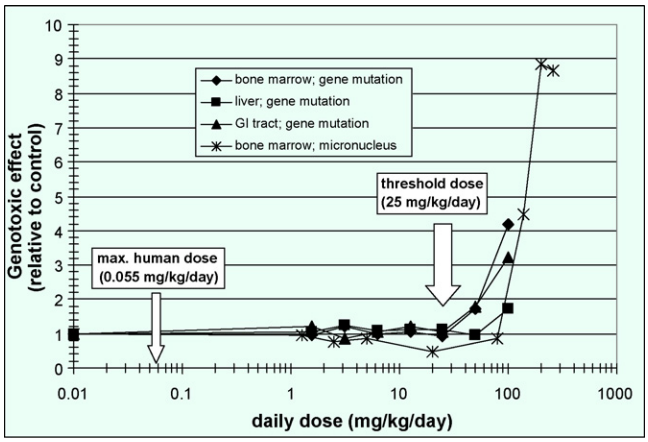

Fig. 1. Dose based comparison of genotoxicity data: graphical presentation of the dose–response relations in the MNT and MutaMouse studies (Gocke et al., 2009c). The relative increase of the genotoxic effect over incidences recorded in the negative control (normalized to 1) animals is plotted as a function of the daily dose.

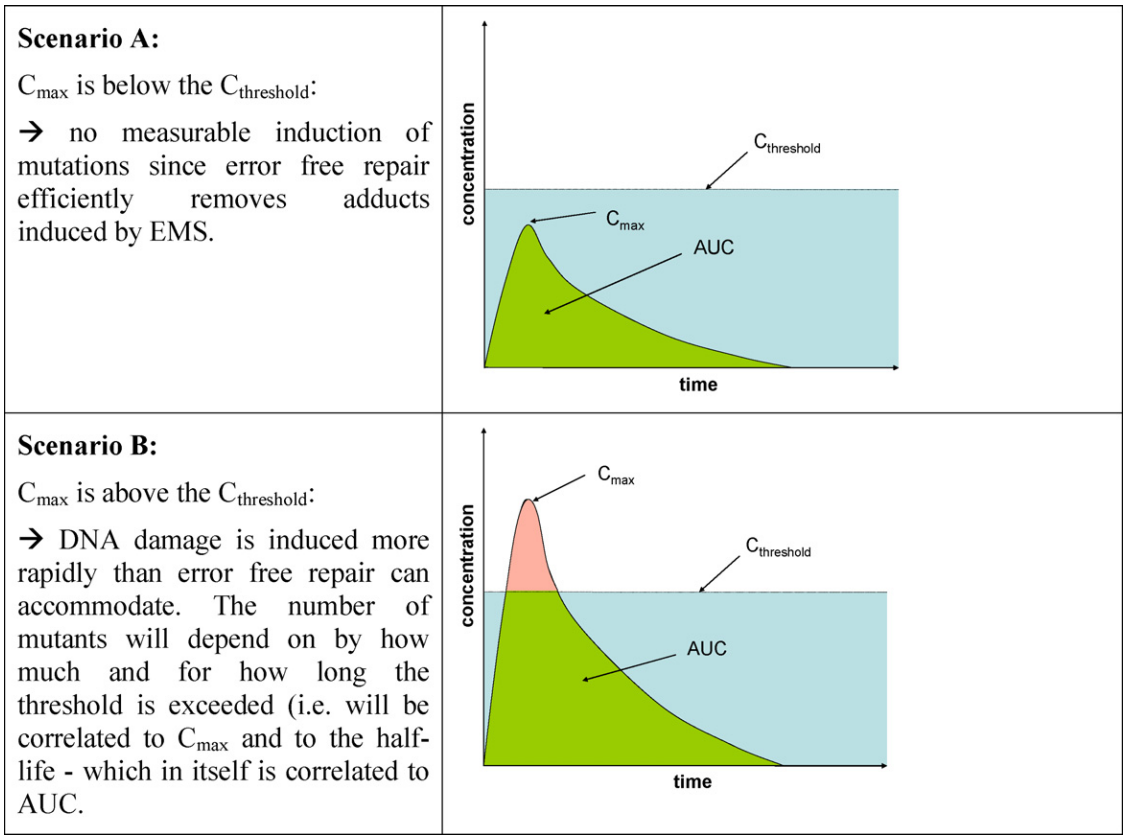

Fig. 2. Consideration of saturation error free repair related to C_{max} and AUC.

In the context of these steps, two major determinants are of critical importance:

(1) The distribution volume of EMS. This factor guides the calculation of the human C_{max}.
(2) The Clearance of EMS which is the major determinant for the calculation of the human AUC.

In principle, the amount rate of DNA damage induced by a genotoxic agent in the DNA of an organ at the time t is proportional to the concentration (C_t) at the target site. So over time the amount of induced DNA damage is proportional to the exposure integrated as a function over time ($= \int C_t \, dt = AUC$).

Since the occurrence of mutations will basically be a function of the amount of DNA damage inflicted during the treatment period, the total exposure in terms of AUC appears to be the logical factor for risk assessment. However, concurrently the DNA damaged sites are repaired by the repair machinery. The thresholded dose–response for EMS provides confidence that a dose was established below which it is exceedingly difficult to demonstrate any differences between mutations in dosed animals and the corresponding controls. This could imply that up to a certain level of EMS DNA damage the repair is error free and dissolves in the background of spontaneously occurring mutations, which partly arise due to error-prone repair mechanisms. Only above the threshold will mispairing and error-prone mechanisms lead to induction and accumulation of mutations.

Whether error free repair is saturated or not will primarily depend on whether the concentration of EMS exceeds a critical value (termed $C_{threshold}$). For this reason, the C_{max} can be one important parameter for induction of mutations. This relationship is depicted in Fig. 2. However, in reality the relationship between time and threshold is likely not a constant one but depends on many factors such as on the re-supply of repair molecules and the time a cell is given to repair prior to the next round of replication or cell division. Saturation of error free repair is obviously governed by the number of repair molecules available and by their enzymatic speed. The number of repair enzymes might vary over time. For instance, the level of the suicide repair MGMT enzyme will initially decrease after genotoxic challenge, but subsequently might surpass initial levels because of induction/adaptation. Hence, the straight line given for the $C_{threshold}$ in Fig. 2 is idealized. In reality, a more complex relationship between insult (EMS alkylation), time to repair (disappearance and renewal of repair molecules) and cell cycle progression does likely exist. However, if repair molecule renewal does not get into conflict with the half-life of EMS to continually alkylate DNA after a single daily exposure as occurring in the Viracept case (the daily dose of 3 g or 10 tablets is recommended to be taken in one go), a deviation from the straight line will not affect much the model presented in Fig. 2. It is also assumed that there will be usually enough time for repair within a cell before it enters the S-phase and alkylations can be turned into mutations. For carcinogenesis, the widely accepted paradigm says that all of the necessary errors in the DNA must occur in the stem cell population of a tissue (Greenfield et al., 1984). The threshold observed in our experiments fulfils all statistical conditions to refute the assumption of linearity (Gocke and Wall, 2009). Hence, we can likely speak of a highly reliable threshold at which no stem cells have proceeded with unrepaired EMS-related alkylations through the cell cycle (Cohen, 2008). For risk assessment, it is important to consider that any mutations observed at the spontaneous background have a similar chance to initiate a tumour process (Cohen, 2008). It is also important to note that this mutation background is partly based on the consequences of endogenously produced methylations at the same sites at which EMS creates ethyl adducts.

2.2.1. C_{max} based risk assessment for EMS in Viracept

A C_{max} based safety factor is a valuable guide for risk assessment especially if no accumulation of a mutagenic effect occurs. In the case of EMS it can be argued that the damage exerted by a single dose below the threshold is completely removed. This is supported by our experimental design, which shows that a fractionation of a total dose into smaller doses given over 28 days abolishes the mutational effect of EMS in bone marrow liver and the GI-tract, whereas a dose fractionation of ENU resulted in evidence for full additivity (Gocke et al., 2009c). Hence, for EMS, the 28 days threshold will unlikely change (i.e. lower) with a continuation of dosing. Consequently, the C_{max} at the threshold for EMS is considered to be an important parameter for risk assessment.

The C_{max} levels of EMS at the Th_D dose in the treated mice has been determined to be

C_{maxThD} = 315 μM	C_{max} values were determined based on single dose pharmacokinetic data using radioactive EMS and on of the levels of ethyladducts in terminal valine of hemoglobin measured in the genotoxicity experiments. These data were employed to calculate the C_{max} at the Th_D dose (Lave et al., 2009a,b)

The C_{max} at the maximal dose of EMS ingested by the Viracept patients was estimated to be

$C_{max\,Hmax}$ = 0.85 μM	C_{max} is primarily related to the volume of distribution and only marginally influenced by the clearance (respectively $T_{1/2}$). In effect, C_{max} is largely proportional to the dose in mg/kg for all species, since for a compound like EMS the volume of distribution does not vary to any larger extent. Thus, the human C_{max} at the dose of 0.055 mg/kg can be estimated with high confidence (Lave et al., 2009b)

Consequently the safety factor based on comparison of the C_{max} levels amounts to

$$SF_{C_{max}} = \frac{C_{max_{Th_D}}}{C_{max_{H\,max}}} = 315\,\mu M/0.85\,\mu M = 370$$

Fig. 3 displays the effects observed in our genotoxicity studies in terms of increase relative to the controls plotted against the C_{max} for EMS. The C_{max} estimate in human is very robust as this parameter depends solely on volume of distribution (Lave et al., 2009b). Since the body-weight normalized volume of distribution was constant across species, it is reasonable to assume that the volume of distribution in man will be similar to the volume of distribution in the preclinical species (Lave et al., 2009b). Hence, the C_{max}/dose unit is

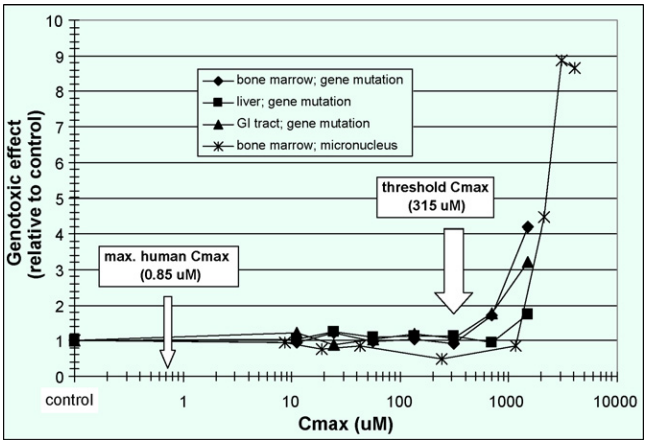

Fig. 3. Concentration (C_{max}) based presentation of the *in vivo* genotoxicity data for EMS: Graphical presentation of the dose–response relations in the MNT and MutaMouse studies. The relative increase of the genotoxic effect over background incidences (normalized to 1) is plotted as a function of the C_{max} value.

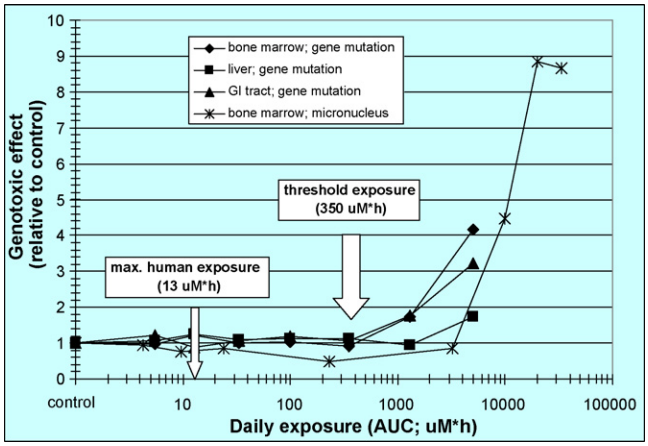

Fig. 4. Exposure (AUC in μM h) based comparison of genotoxicity data: graphical presentation of the dose–response relations in the MNT and MutaMouse studies. The relative increase of the genotoxic effect over the background incidences (normalized to 1) is plotted as a function of the daily exposure.

rather similar across species. The confidence in this calculation is high.

2.3. Total exposure (AUC) based risk assessment for EMS in Viracept

As the exact kinetics of removal of EMS alkylations in the DNA after a daily insult are not known, the presence of EMS to the target molecules over time could also be a factor for risk assessment. Hence, also an AUC based safety factor has been calculated. Based on the DMPK characteristics of EMS (Lave et al., 2009a,b) we have been able to predict the pharmacokinetic parameters for EMS in man. Based on these data, we calculated the exposure (AUC in μM h) in man at the maximal dose experienced by the Viracept patients in order to set this value in relation to the AUC at the threshold (NOEL) for genotoxicity in mice.

The exposure (AUC) levels of EMS at the t_D dose in the treated mice has been determined to be

E_{ThD} = 350 μM h	Exposure of the mice was determined based on single dose pharmacokinetic data using radioactive EMS and on of the levels of ethyladducts in terminal valine of hemoglobin measured in the genotoxicity experiments. These data were employed to calculate the AUC at the Th_D dose (Lave et al., 2009a,b)

The maximal exposure of the Viracept patients was estimated to be

E_{Hmax} = 13.0 μM h	For this calculation the EMS pharmacokinetic profile was scaled from mice, rat and monkey to man. Furthermore *in vitro* data on chemical stability were considered. The human exposure at the 0.055 mg/kg dose was then calculated (Lave et al., 2009b)

Consequently the safety factor based on comparison of the exposure levels amounts to

$$SF_{exposure} = \frac{E_{ThD}}{E_{Hmax}} = 350\,\mu M\,h/13.0\,\mu M\,h = 28$$

Fig. 4 displays the effects observed in our genotoxicity studies in terms of increase relative to the negative controls plotted against the AUC for EMS. Regarding the accuracy of this prediction, the following reasoning can be provided: The main factor that determines AUC (i.e. how long EMS will remain in the body to react with targets after an oral ingestion) is the elimination from the body via chemical degradation, metabolism, hydrolysis, diffusion, active

transport, etc. The elimination of EMS is non-linear in mice i.e. rapid at low doses of 1–5 mg/kg and with tendency to slower elimination (translating into longer half-life) at higher doses (25–50 mg/kg) that produce mutations in mice (Lave et al., 2009a). Elimination is prolonged in rats and monkeys, compared to mice (Lave et al., 2009a). For purposes of estimating the EMS exposure in humans, two different approaches were used (Lave et al., 2009b). In one approach, the exposure was calculated with the slowest possible elimination based on stability of EMS recorded in buffer solution. Spontaneous breakdown of EMS occurs with a $T_{1/2}$ of ~11 h. Consequently, the calculated human AUC is the worst case upper estimate. Since every biological system will most likely degrade and eliminate EMS faster than buffer, the real human AUC for EMS is likely lower than that presented here. Indeed, if the degradation in a biological system would be identical to degradation in buffer, it would mean that there is no reactivity with the biomolecules, i.e. there could be no biological effect, hence the chemical in question would not be able to exert a mutagenic effect. In the second approach allometric scaling was done using the scaling factor of 12 for scaling from mouse to man. This approach yielded, incidentally a very similar AUC than the calculation based on chemical degradation (Lave et al., 2009b).

3. Calculation of DNA ethylations at threshold dose

Based on the data published by Murthy et al. (1984) we can estimate the number of ethylations present in the mouse liver at the threshold dose of 50 mg/kg. Murthy et al. (1984) determined the degrees of N7-Gua ethylation in mouse liver and kidney and also the degree of total ethylation in hemoglobin for single doses of EMS between 0.04 mg/kg and 446 mg/kg. As described in Gocke et al. (2009c) our data on ethylation of globin agree very well with the data published by Murthy et al. Therefore we can assume that the amount of DNA ethylation in our experiments also conform to the data of Murthy et al. (1984).

In the report on the genotoxicity studies (Gocke et al., 2009c) we have calculated the daily increment of ethylvaline adducts. At the dose of 50 mg/kg/day we determined a daily increment of 8.5 nmol ethylvaline/g globin (for details see also Fig. 9 in Gocke et al., 2009c). Murthy et al. (1984) determined that the total globin adduction rate was found to be 15 times higher than the adduction rate at terminal valine (1.5×10^{-4} versus 0.1×10^{-4} $1 \times g\,Hb^{-1} \times h^{-1}$).

Thus, for total globin ethylation we derive a value of $8.5 \times 15 = 128$ nmol/g globin at 50 mg/kg. Fig. 5 shows a plot of the levels of N7-Gua ethylation in liver and kidney in relation to total globin ethylation in EMS treated mice (data taken from Murthy et al., 1984). We can see that globin ethylation of 128 nmol/g globin corresponds to a DNA ethylation of 70 nmol N7-Gua/g in the liver. With an average molecular weight of a nucleotide of 309 we calculate that 1 g DNA amounts to about 0.0032 mol of nucleotides. Thus we obtain 70 nmol N7-Gua/0.0032 mol nucleotides = 2.2×10^{-5} N7-Gua ethylations per nucleotide.

The haploid mouse genome contains 5×10^9 nucleotides. We, therefore, derive a value of 230,000 N7-Gua ethylations induced by 50 mg/kg in the diploid mouse liver cell. Since N7-Gua ethylations amount to about 60% of total ethylations (see Table 2 in Beranek, 1990) the total DNA ethylation at this dose is estimated to be 380,000.

In conclusion, we estimate the every mouse liver cell can repair 380,000 ethylation adducts induced daily by 50 mg/kg of EMS in its genome without making errors leading to an measurable increase of mutations and a chromosomal aberrations.

We are aware of the fact that ethylations of globin are often used as biomarker for risk calculation with exposure to genotoxic carcinogens (Törnqvist et al., 1988, 2002). This may be justified in situations, in which adducts in proteins, adducts in DNA, mutations and cancer follow comparable dose–effect relationships and can be considered cumulative. In the case of EMS though, a linear accumulation of ethyl adducts to the terminal valine of globin have been measured by us already at dose levels with do not yield evidence for induction of mutations. Our adduct data with globin are consistent with the data of Murthy et al. (1984), who show roughly linear dose relation for globin as well as DNA alkylation after EMS treatment. Hence, we state that in our case, ethyl adducts in globin may be used as indicators of exposure rather than risk. We have shown that the elimination of ethyl adducts in globin follows the very long half-life of globin (Lave et al., 2009a). There is no active process of removal of such protein adducts. Adducted proteins are not repaired but DNA is, which implies that determination of DNA adducts in steady state with chronic intake is a better marker of risk than protein adducts, if the relationship between DNA adducts and mutational effect is known. Hence, contrary to globin, our mutation threshold data imply an active and likely error-free removal of ethyl adducts from the DNA thus constituting a threshold for resulting DNA damage and no accumulation of effect over time.

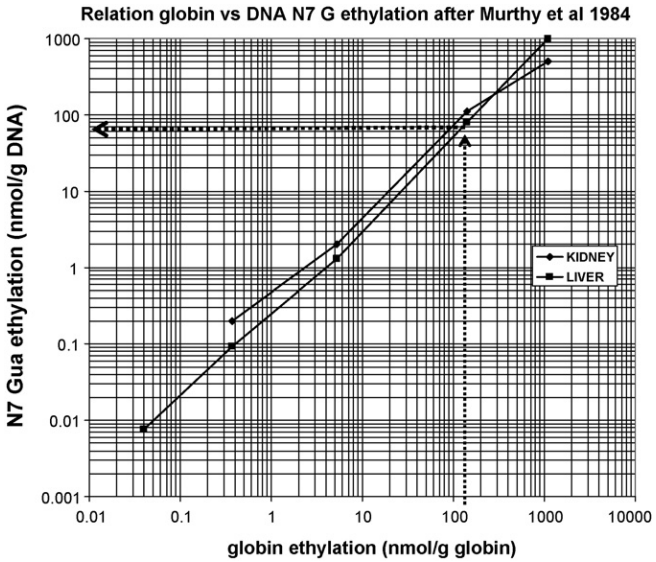

Fig. 5. Ethylation by EMS in liver and kidney DNA (N7 Gua) versus total ethylation of hemoglobin.

4. Factors to consider in the context of possible ingestion of EMS by HIV infected patients having taken Viracept

Since a pharmaceutical is used in a human disease context, there is a need to place the previously elaborated generic risk assessment for EMS for a prototypic human individual into the very specific disease conditions of HIV infected patients, for which Viracept is used. The viral infection could have an impact on the ability to repair damage inflicted by EMS. Further, these patients usually take a variety of other medications, which could impact on the toxic effect of EMS. In the following, an analysis of these situations is provided to approach a comprehensive risk assessment for the likely affected patients.

4.1. Species and tissue comparison of the DNA repair molecule methyl guanine methyl transferase (MGMT)

EMS induces no 'unusual' DNA damage (Mientjes et al., 1998), consting almost exclusively of GC → AT transitions (Op het Veld et al., 1997). Various endogenous and environmental agents alkylate DNA at the identical places where EMS attacks. Chen et al. (2007) find low, but measurable levels of 7-ethyl-Gua (the pre-

dominant DNA alkylated base after EMS exposure) in the DNA of human liver samples. They conclude "*These results demonstrate that 7-ethyl-Gua is a common DNA adduct in human liver with likely endogenous sources. . ..*" Even higher levels of 7-methyl-Gua, are observed (De Bont and van Larebeke, 2004). This lesion results mainly from the endogenous methyl donor S-adenosyl-methionine (SAM). Another, similar DNA lesion is 7-(2-hydroxyethyl)-Gua, which results from endogenous ethane and exogenous ethylene oxide. Further, cigarette smoke is known to contain alkylating agents (Singh et al., 2005; Carmella et al., 2002).

All of these alkylation damages are repaired by several, overlapping repair mechanisms (MGMT, base excision repair, nucleotide excision repair, mismatch repair). Hence, our DNA repair system is evolutionary prepared and adapted to remove such DNA damage – at least at low levels – with a high efficiency and a low error rate. In terms of the fundamental rate limiting steps of DNA repair that are important for an apparent threshold for mutation induction, the DNA repair protein MGMT is known to be a key tool in error-free removal of alkylations, in particular at the O^6 of guanine, a target of alkylation for EMS. This proof-reading is required from an evolutionary perspective since, as noted above, there are ubiquitous endogenous and exogenous sources of such alkylations for mammals and consequently humans.

Because of the importance of O^6 alkylations for mutation generation, the possible impact of species and tissue differences as well as inducibility of especially MGMT on the extrapolation of the thresholds for mutations and chromosome damage in our present studies to humans in general and the HIV target population specifically is presented in the following.

4.2. Literature data on MGMT pertinent to the "EMS in Viracept" case

There is extensive literature on the DNA repair protein, O^6-methylguanine methyltransferase (MGMT), including reviews on its action, cellular content, inducibility and genetics (see e.g. Kaina et al., 2007). MGMT is also referred to as alkyl guanine transferase (AGT) as it can remove ethyl groups as well as methyl groups. Is can be reasonably argued that the main evolutionary purpose of this molecule is to remove endogenously occurring methyl-adducts at the guanine site and to a lesser extent at the thymine site.

MGMT is a highly conserved DNA repair molecule in mammalian species. It has arguably evolved as a consequence of the permanent endogenous (and exogenous) alkylation burden to DNA. One of the main endogenous sources of methyl-adducts of haemoglobin, and also DNA, is S-adenosyl methionine (Törnqvist et al., 1988). While the source of ethyl-adducts has not been conclusively identified, ethanol and its metabolite acetaldehyde are speculated to be factors (Fang and Vaca, 1997; Chen et al., 2007). The background levels of O^6-methylguanine are orders of magnitude higher than those of O^6-ethylguanine in humans (Swenberg et al., 2008).

MGMT removes methyl and ethyl groups from altered DNA bases in an error-free way by reverting altered bases to their original structures. MGMT transfers the alkyl-group onto a cysteine in its catalytic pocket. A single MGMT molecule removes a single alkyl group. Since alkyl-MGMT is not regenerated, the number of MGMT molecules in a given cell and their generation is likely directly proportional to the cell's ability to handle O^6-alkylations.

Consequently, Lutz (1982) calculated the number of repair units in a given cell that would be needed to handle DNA alkylations and described how the kinetics of generating new MGMT molecules would influence the ability of cells to remove alkylations before saturation takes place (and mutations are induced). These data suggested that the hamster liver has a 50% lower capacity for DNA repair of O^6-methylguanine than rat liver.

Differences in levels of MGMT between species and tissues are known with low levels generally reported for brain tissue and haematopoietic progenitor cells (bone marrow) in rodents and humans (Gerson et al., 1985). Humans appear to have higher MGMT activity than rats or mice (Grafstrom et al., 1984). Gerson et al. (1986) conclude on their studies on species and tissue comparison of MGMT: "*These studies suggest that mouse and rat tissues should be more susceptible than human tissues to the carcinogenic action of agents whose mutagenic potential is mediated by formation of O^6 alkyl-guanine adducts. Although mouse and rat may be sensitive indicators for determining the carcinogenic potential of an agent, mutagenesis in human tissues is not likely to occur at the same dose level as in the mouse and rat since human tissues contain higher levels of O^6-alkylguanine-DNA alkyltransferase.*" When comparing species with respect to MGMT activity relative to DNA content for each tissue tested, humans rank highest followed by rats and mice (Gerson et al., 1985, 1986; Grafstrom et al., 1984). Overall, these data imply a higher sensitivity of mouse tissue to the mutagenic activity of alkylating agents than human tissue.

Regarding correlation of MGMT activity with outcome (mutations and cancer), the persistence of DNA adducts in brain tissue of rats has been correlated with low levels of MGMT (Zagon and McLaughlin, 1981; Jun et al., 1986). In this context, it is interesting that the primary location of tumours induced by high intravenous doses of EMS and MMS in rats treated transplacentally and postnatally were neurotropic brain tissue tumours (Schneider et al., 1978). It may be concluded that this organotropy reflects the high mutational load in these tissues at exposure levels in excess of the MGMT repair capability. There appears to be a striking similarity in these aspects to human brain cells. Various investigations have shown that the response of human glioblastomas to chemotherapy with polyfunctional alkylating agents (see e.g. Wiewrodt et al., 2008) is associated with the MGMT levels in such tumours. Tumours with a low MGMT level (as seemingly common for brain tumours) appear to be more susceptible to treatment with alkylating agents than tumours with higher levels (Chinnasamy et al., 2004; Kaina et al., 2007; Wiewrodt et al., 2008). Consequently, lowering of MGMT levels in human tumours has been discussed as a therapeutic approach in general to enhance the success of cytostatic therapy (Barvaux et al., 2004). Also, the selection of MGMT mutant cells with a high resistance to alkylating agents (due to induction and selection for high MGMT levels) has been proposed as a human transplantation approach for repopulating haematopoietic cells *in vivo* in HIV patients (Davis, 2004). Such cells are protected from resistance against DNA alkylation damage induced by BCNU, a polyfunctional alkylating agent with much higher mutational potency than EMS, as a consequence of induction of MGMT. In translating the evidence to EMS, these data fully support recently obtained data by Doak et al. (2008) who reported that low concentrations of MMS induce MGMT *in vitro* in the human lymphoblastoid cell line AHH-1.

In the context of human polymorphism for MGMT, studies conducted by Jiao et al. (2007) did not yield any conclusive evidence for a direct correlation between MGMT functionality and ENU-related DNA damage in human lymphocytes, *ex vivo*. The authors speculate that the variation in both expression and activity of MGMT could mask any minor effects caused by a certain polymorphism. This is supported by Kaina et al. (2007), who report that MGMT is inducible by several exogenous factors such as caffeol and cafestrol in coffee, tea polyphenols and corticosteroids and endogenous factors such as chronic inflammation. Beta-interferon has been shown to down regulate MGMT. It is also reported that MGMT activity is increased in the placenta of smokers (Slupphaug et al., 1992).

In the context of applicability of the above referenced data for the threshold data on EMS in animals and their human relevance in general and for Viracept patients in particular, the following notions are put forward:

(1) It is interesting to know that the AHH-1 cell line, in which Doak et al. (2007) have reported conclusively about thresholds for mutation induction by MMS and EMS are derived from Epstein–Barr transformed human B-lymphocytes. The MGMT level of this cell type is not known, yet an Epstein–Barr transformation seems not to abolish the threshold for MMS and EMS.
(2) The mutation threshold of 25 mg/kg/day observed in bone marrow of transgenic mice in Roche's new studies is based on steady state for mutations in haematopoietic progenitor cells that are known to possess low levels and activity of MGMT (Gerson et al., 1996).
(3) Humans appear to have higher MGMT activities than rats and mice (Gerson et al., 1985).

In conclusion, the above data imply a likelihood of variations of repair ability of different tissues or species to remove EMS alkylations. It can also not be excluded that MGMT levels are lower in HIV infected individuals compared to non-infected humans. While we have no data to show a mutation threshold in HIV infected cells or in humans, the presence of a mutation threshold in the bone marrow, a tissue with low levels of MGMT, and in the mouse, a species with low MGMT activities compared to humans, is reassuring in case the HIV infection as such would lower the MGMT levels and would thereby constitute a lower mutation threshold than in non-infected humans.

4.3. Potential impact of the disease status of Viracept patients

An immuno-compromised state, as experienced by AIDS patients in progressed disease stages, is known to enhance tumourigenesis (Everly et al., 2007). However, this mode of action is involved in the late steps of the cancer process, i.e. tumour promotion and metastasis, and has no connection to the early stages of tumour development (mutagenesis, tumour initiation), in which the DNA damaging property of EMS plays a role.

HIV infected patients may be not only impaired in their immune system but many of them certainly do also show organ malfunctions due to other related or unrelated diseases or conditions, especially as Viracept has been taken in many countries with low hygienic standards. In the following, some arguments are provided as to why we think that such conditions do not have any impact on the safety factors calculated:

(1) *Impairment of GI tract function*: Local effects and the systemic availability of drugs can be influenced by GI tract malfunctions such as diarrhoea, constipation, malabsorption or inflammatory lesions. These may not only decrease but can also increase systemic availability of agents. Hence, further to direct analysis of mutations by EMS in the GI tract, we have assumed ~100% bioavailability from the GI tract for our risk assessment. Hence, for this assumption, any increase in bioavailability of EMS by changing conditions in the GI tractwill not affect our calculated safety factors.
(2) *Renal insufficiency*: Renal excretion of EMS occurs via methane sulfonic acid (Roberts and Warwick, 1958). Since the main clearance (in the mouse) is driven by hydrolysis and exhalation (Roberts and Warwick, 1958), the kidney has little impact on the clearance of EMS (Lave et al., 2009b). Further, we assumed the half-life of EMS in humans to be similar to that in PBS buffer. Hence, if the half-life of EMS would be still potentially affected by renal insufficiency, this is taken care of by the risk assessment presented.
(3) Hepatic impairment: Our *in vitro* studies with liver microsomal preparations of various species have shown that EMS in highly stable in such systems (Lave et al., 2009a). Hence, there is little evidence for microsomal metabolism of EMS. From these data we can infer that the main metabolism, or perhaps more correctly—degradation pathways for EMS *in vivo* are likely of cytosolic nature. These are not confined to the liver. Hence, conditions of liver impairment are covered by the risk assessment presented.

4.3.1. Ability of Viracept patients to repair EMS lesions

In principle, the HIV-virus can insert itself into any place of the genome (Uren et al., 2005), and thus, may hit upon a DNA repair gene, whose product is involved in the elimination of the DNA damage inflicted by EMS. Thereby, a reduced repair competency could be effected in the respective cell. However, this is a random and not a directed process and the likelihood of insertion into one of the responsible genes, such as MGMT (O^6-methyl-guanine DNA methyl transferase, is extremely low (likely in the order of 1 in a million). Additionally, there will still be the second allele on the homologous chromosome which will not be compromised by the insertion. Furthermore, the DNA damage inflicted by EMS can be repaired by different repair pathways, which are partly overlapping in their repair capabilities.

4.3.2. (Epigenetic) influences of HIV infections

Viral attack and cellular defenses are obviously opposite forces during infection. On the one hand viral infection perturbs the regulatory circuits of the cell. On the other hand, cellular defense mechanisms attempt to hinder the expression of the viral genome. Retroviruses can stay in a dormant state (latency) in mammalian cells. During this period the silencing of the viral genes appears to be linked with an active, epigenetic process, namely the methylation of CpG dinucleotides (Gutekunst et al., 1993). De novo methylation has been observed in the viral promoter regions after infection, thus shutting down their transcription. The responsible enzymatic process is performed by the mammalian DNA methyltransferases (not to be confused with the MGMT enzyme).

There are a few reports that upon viral infection the 'methylation defense' is not fully confined to the viral genome but is also occurring in endogenous (cellular) genes. Specifically, methylation of CpG islands in the promoter of the p16 (INK4A) gene has been observed after infection of lymphoid cells with integration-defective HIV-1 viruses (Fang et al., 2001) and HBV/HCV infected cirrhotic liver cells (Kaneto et al., 2001). The methylation of the p16(INK4a) gene, however, seems to be a specific response on the regulatory circuit involving G1-cyclin dependent kinases (CDK's) (Yeung et al., 2005). Thus, this endogenous methylation might be explained as a secondary effect of viral infection on cell cycle control. No such explanation could be logically applied to a repair gene activity. And indeed, methylation of the MGMT gene has not been observed in SV40 induced tumour cells (Amara et al., 2007), and in hepatitis B induced hepatocellular carcinoma cells (Su et al., 2007). In another study, methylation increased with progression from normal liver tissue to chronic hepatitis to liver cirrhosis to hepatocellular carcinoma (in line with the processes of carcinogenesis and uncontrolled invasive cell growth) while at most a weak correlation was seen between MGMT-gene methylation and hepatitis viral infection (Matsukura et al., 2003).

In this context, it is to be noted, that silencing of tumour suppressor genes by methylation is one of several genetic or epigenetic modes of gene inactivation mechanisms in tumour development. The repair gene MGMT can be considered as a tumour suppressor gene. It is inactivated in many tumours, also by means of CpG methylation (Esteller and Herman, 2002). Relevant for our discussion is the observation that for immunodeficiency related lymphomas (one of the prominent tumours in HIV patients) it has been reported that the MGMT promoter was less frequently hypermethylated in HIV immunosuppressed cancer patients than in post-transplant-immunosuppressed patients (Rossi et al., 2003).

This observation supports that the viral infection has no causal role in epigenetic silencing of the MGMT repair gene.

There is accumulating evidence that cancer patients with human immunodeficiency virus-1/acquired immunodeficiency syndrome (HIV-1/AIDS) have more severe tissue reactions and often develop cutaneous toxic effects when subjected to radiotherapy. In this context, it appears that the HIV-1 Tat protein interferes with the ability of the infected cells to handle DNA double strand breaks as induced by γ-radiation (Sun et al., 2006). Tat promotes proliferation in various cell types by interacting with different host proteins. Overexpressed cyclin B1 induced by Tat might be another factor promoting cell growth (Sun et al., 2006). Since such double strand breaks are not induced by EMS, an interaction by this mechanism in HIV infected cells would not be expected.

It is concluded that there is no experimental evidence that HIV viral infection, per se, induces an increased susceptibility of the Viracept patients to the genotoxic mode of EMS toxicity.

4.4. Potential impact of medications taken by Viracept patients

Antiretroviral therapy with nucleoside analogues such as AZT, DDC leads to elevated chromosomal aberration frequencies in AIDS patients and in not infected newborns (Witt et al., 2007; Walker et al., 2007). Nucleosidic reverse transcriptase inhibitors (NRTI's) are thought to be especially mutagenic in mitochondria due to the higher sensitivity of the archaic mitochondrial DNA polymerase whereas the mammalian specific polymerases are much less affected. In contrast, EMS does not have a mutagenic potential in human mitochondria *in vitro* (Mita et al., 1988) although it cannot be excluded that methodological limitations prevented their detection.

There are various hints that antiretroviral therapy that include nucleoside analogues such as AZT can be considered as carcinogenic in humans. Especially, antiretroviral drugs used to prevent HIV transmission from mother to child can cause genetic damage in infants that may increase their risk of developing cancer. Combinations, typically including the nucleoside analogue zidovudine (AZT, Retrovir), have reduced the mother-to-child transmission of HIV from around 25% to less than 2% in infants who are not breast-fed. Pregnant women with HIV in developed countries receive antiretroviral treatment as standard care during pregnancy and infants are given zidovudine for 6 weeks after birth. NRTIs can incorporate into the DNA of human cells, raising concerns that this may have future health consequences. In one animal study, AZT was dosed to female mice and rats during the last week of pregnancy and the offspring was evaluated for tumour presence 2 years later. An increase in haemangiosarcoma in male mice and leukaemia in female rats was found (Walker et al., 2007). There was also some evidence of increased risk of liver cancer and glioma—cancer of the central nervous system. The second animal study looked at the offspring of mice given AZT during pregnancy. The researchers found genetic mutations in two genes – K-ras and p53 – which are associated with lung cancer in humans. The rates of lung cancer were significantly higher in those mice whose mother had been given the highest doses of AZT (Hong, 2007). The authors of this study said that their data suggest that infants exposed to AZT in the womb may be at increased risk of cancer as they age.

In two human studies, evidence of DNA damage in the blood of infants born to mothers who had been taking antiretroviral therapy. In the first the researchers studied immature blood cells in the blood of both the newborns and their mothers for the presence of micronuclei. A 10-fold increase in the number of micronucleated blood cells–in both the infants and the mothers who had been taking antiretrovirals. But the frequency of these abnormal blood cells dropped in the first 6 months after birth to normal levels (Witt et al., 2007). In the second human study researchers looked at a gene involved in building red blood cell walls, glycophorin A (GPA), in the blood of children born to women who had been taking both zidovudine and lamivudine (*Epivir*). They found GPA damage was more likely in the children of mothers who had taken both drugs, and these mutations were still present a year after birth (Escobar et al., 2007). These data show that many of the normally used medications for HIV patients bear their own risks of damage to the genetic material. The molecular nature of the damage is very different from that of EMS and will not influence the repair of EMS-related DNA damage. Further, lack of metabolism of EMS by microsomal enzyme systems (Lave et al., 2009a) indicates that there would be no interference with medications that undergo microsomal metabolism. Further, for human exposure simulation it was assumed that EMS has a half-life similar to that in PBS buffer, i.e. 11 h. This means that by no means, there is a possibility for an even longer half-life of EMS in any human compartment. In addition, impairment of clearance and metabolism processes in the liver or kidney of affected patients cannot create a higher exposure in any individual patient than the calculated C_{max} and AUC.

As a further possibility of interaction, the notion that hypersensitivity adverse drug reactions are much more common among patients with acquired immunodeficiency syndrome (AIDS) than in the general population is important. High rates of hypersensitivity reactions to clindamycin have been noted (Wijsman et al., 2005). Further, In HIV-infected patients treated with sulphamethoxazole-trimethoprim, rates of adverse drug reactions have been reported to reach 80% and are commonly in the range of 30–40% (van der Ven et al., 1994). In this context, Wijsman et al. (2005) have reported an increased formation of reactive metabolites from clindamycin and sulphamethoxazole (SMX) in human immunodeficiency virus (HIV)–infected MOLT3 cells. As EMS is a directly acting agent, any such potentiation of its effects by such processes to enhance the formation of reactive metabolites of drugs in HIV infected cells will not impact on the genotoxicity of EMS.

Hence, overall there is no evidence that other medications taken by Viracept patients have any impact on the toxicity of EMS and will not influence the threshold for mutation induction, teratogenesis and cancer.

4.5. Sensitivity of embryonal versus adult tissue towards the alkylation potential of EMS

Viracept is also taken by HIV infected pregnant mothers. Hence, any risk assessment would be incomplete without addressing teratogenicity of EMS. There is no doubt that EMS is a teratogenic agent. It is acknowledged that its teratogenicity occurs downstream of its potential to alkylate DNA (reviewed in Gocke et al., 2009a). Experimental data support the assumption that rodent embryonal cells may be at a lesser risk for alkylation of DNA and their mutational consequences by EMS than the maternal tissue.

Since MGMT is such an important molecule protecting DNA against exogenous and endogenous alkylation, it is prudent to ask how embryonal stem cells or differentiating cells in foetuses may behave relative to cells of the adult organism. In this context, Roos et al. (2007) reported an exquisite ability of MGMT in non-differentiated mouse embryonal stem cells (EST) and differentiated mouse ESTs to remove DNA adducts. He also observed that ESTs undergo more readily apoptosis and are thus not propagated when DNA damage exceeds MGMT repair capability versus normal mouse fibroblasts. They concluded "*that EST cells have a fine-tuned system aimed at protecting them from mutations provoked by O^6-methylguanine DNA adducts induced endogenously or exogenously by methylating genotoxins that are ubiquitously present in the environment*."

In the context of risk assessment for EMS in Viracept if it were taken by pregnant women, the potential existence of a higher effi-

ciency of cellular repair (and apoptosis of more damaged cells) in embryonal cells versus the maternal organism is supported by several lines of evidence:

(1) The NOEL for morphological changes in mouse embryos for EMS was 100 mg/kg and the lowest effective dose was 150 mg/kg intraperitoneally (Platzek et al., 1995) and hence is much higher than the threshold for mutation induction of 25 mg/kg/day in adult bone marrow and GI-tract tissue of transgenic mice.
(2) O^6-ethylG and N^7-ethylG DNA adduct levels were determined in mouse embryonal tissue by Platzek et al. (1994). There was linearity for adducts in maternal liver and embryonal tissue in the teratogenic dose range (137–250 mg/kg). In the subteratogenic dose range (45.1–97.6 mg/kg), adducts in embryonal tissue were found but a flatter (sublinear) dose-to adduct relationship exists. However, Murthy et al. (1984) observed a linear dose-to N^7-ethylG adduct relationship in kidney and liver of adult mice in this dose-range. Robbiano et al. (1989) reported a lower covalent binding index to DNA in liver, brain and kidney tissue of rat fetuses in comparison to the respective tissues in the dams. Lack of data does not facilitate a direct comparison of adduct levels in fetal and maternal blood though to potentially judge more directly any effects of the placenta on the materno-fetal transfer of EMS.
(3) A dose of 100 mg/kg EMS given intravenously was needed to yield evidence for transplacentally induced tumours in rats (Schneider et al., 1978). Taking into account that EMS does have a longer half-life in rats than in mice (Lave et al., 2009a), this dose is likely to lead to an exposure by an order of magnitude higher compared to the exposure at the threshold dose of 25 mg/kg/day in mice.
(4) Finally, a study on mouse embryonal stem cells (EST) cells (Clemann, 2008) yields evidence that EMS had a very low cytotoxicity in EST cells with an IC50 of ~300 μg/ml. Inhibition of differentiation was seen with an ID50 of 110 μg/ml and 120 μg/ml in the two experiments. This compares to an IC50 of MMS of 20 μg/ml and an ID50 of 2–3 μg/ml. It can be concluded that EMS has a very low potential to act on differentiation of EST cells and its cytotoxicity is only observed at high concentrations relative to MMS.

It cannot be excluded that some of the published differences in sensitivity between maternal and embryonal tissue with lower sensitivity of embryonal tissue compared to the maternal tissue may be due to limited materno-fetal transfer of EMS. However, the physico-chemical characteristics of EMS would not readily allow the conclusion of a limited transfer through the placental barrier. Overall, it can be reasonably argued that teratogenicity of EMS is an event downstream to the consequences of DNA alkylation and cellular damage and that embryonal tissue shows evidence for less damage than maternal tissue at the same dose levels of EMS. Hence, based on our evidence for a threshold for mutations for EMS in adult mice and the considerations presented above, teratogenic responses towards EMS would not be expected at doses below the threshold for mutations.

4.6. Conclusion on risk assessment for EMS in Viracept based on evidence for a thresholded dose–response

There can be no doubt that EMS is capable of inducing gene mutations and chromosomal aberrations *in vitro* and *in vivo*. Like for other DNA reactive agents it has been generally assumed that EMS induces genotoxic effects in a linear dose–response as predicted by the 'single hit, single target' hypothesis. Since mutations, if occurring in genes promoting neoplastic transformation, have the potential to initiate tumourigenic growth, it has, consequently, been generally assumed that cancer induction by DNA reactive genotoxins would similarly adhere to a linear dose–response relationship.

However, this hypothesis does not take into account the protective mechanisms, such as error proof repair, that might limit (or totally counteract) the induction of mutational changes at dose levels that are below the saturation level of the defense machinery. Thus, for genotoxic agents which induce DNA lesions that can be effectively removed by the repair machinery a sublinear dose–response relationship (i.e. slope increasing with dose) with a 'pragmatic' threshold might well exist regarding the induction of mutations/chromosomal aberrations and, in consequence, also regarding the induction of neoplastic growth.

Alkylation by EMS is predominantly targeting N-atoms and to a lesser extent O-atoms whereas alkylation by ENU is more effective at targeting O-atoms. These lesions have different mutational consequences due to being removed by different repair pathways and due to the mispairing property of O-alkylated guanine and thymidine leading directly to gene mutations, when the replication fork passes such a lesion.

Its is generally acknowledged that for directly DNA damaging agents it is prudent to assume linear dose–response relationships for induction gene mutations and chromosomal aberrations. However, a recent dedicated investigation of the dose–response relationships of several alkylating agents in the low dose range (Doak et al., 2007) strongly supported the notion that this hypothesis can be differentiated depending on the type of DNA lesions induced by the genotoxins–at least for *in vitro* studies with human cell lines. Genotoxins causing only a limited alkylation at O-atoms, a group to which EMS belongs, were observed to induced a 'pragmatic' threshold for induction of gene mutations and chromosomal damage, while agents inducing strong alkylation at O-atoms, such as ENU, were observed to induce the linear dose–response, in line with general expectations. Notably, further analysis of the ENU data (Johnson et al., in press) suggested the presence of a threshold even for ENU. Thus, it may be considered that ENU as the stronger alkylator than EMS was not tested down to low enough concentrations to really recognize the presence of a threshold. It is hard to envisage that the alkylations induced by the tested agents are so fundamentally different to conclude that only EMS and MMS display a threshold and ENU and MNU not. Thus, further experiments are warranted to investigate whether ENU and MNU yield evidence for mutation thresholds, similar to EMS and MMS, albeit at (much) lower doses.

We have now extended the *in vitro* observations from Doak et al. (2007) to *in vivo* studies for gene mutations and chromosomal damage on EMS and ENU. For EMS, we have observed clearly thresholded dose–response relationships for the induction of chromosomal damage in bone marrow with a threshold dose of 80 mg/kg/day, as well as for the induction of gene mutations in bone marrow, GI tract, and liver with threshold values of 25 mg/kg/day and 50 mg/kg/day, respectively. For ENU, we were not able to establish a threshold but we tested only a limited number of doses compared to EMS. Further analyses of the data support the notion that a possible threshold dose of ≤0.5 mg/kg/day for ENU would be compatible with the data (Gocke et al., 2009c). It is noteworthy to point out, that our study results are not in contradiction to the many published studies on EMS (reviewed in Gocke et al., 2009a). The presence of a threshold has not been conclusively recognized so far because the applied dose levels were generally too high and or insufficient analyses have been provided at and below the no effect level. A sublinear dose–response for EMS had, indeed, been apparent in many of published *in vivo* genotoxicity studies (Gocke et al., 2009a).

We contend that the statistical analyses of our data (Gocke and Wall, 2009) provide reliable support for a thresholded

dose–response relationship regarding clastogenic effects in bone marrow and mutagenic effects in three representative organs. We are aware that statistics cannot supply a positive proof. There is also a possibility that for other organs and for other target genes different dose–effect relations could exist. However, we believe, our data are coherent and sufficiently complete to yield a sound foundation for diverging from the default risk assessment approaches based on linear dose relations for the test compound EMS.

For cancer risk assessment, it is also noteworthy that the existing tumour data on adult organisms (mainly rats) and in transplacental approaches (in rats) do not suggest a tumourigenic potential at lower doses than the mouse mutation threshold. EMS induced mainly tumours in mammary and brain tissue as well as the kidney and lung. Their induction required administration of ≥100 mg/kg intravenously in transplacental models. Doses of ~50 mg/kg/day in drinking water for ≥1 month produced mammary tumours (Gocke et al., 2009a,b).

Based on our DMPK assessments (Lave et al., 2009a,b) we can estimate the AUC of EMS in mice. For the dose of 25 mg/kg an AUC of 350 μM h is estimated (Lave et al., 2009a,b). Doak et al. (2007) determined the lowest effective concentration for EMS to be 1.4 μg/ml in their *in vitro* experiments, the threshold concentration was between 1.2 μg/ml and 1.3 μg/ml for gene mutations and chromosomal aberrations, respectively. The cells were treated for 24 h. For buffer and serum we have determined the half-life of EMS to be about 11 h in serum. On this basis, it is possible to calculate the AUC of EMS for the *in vitro* exposure conditions employed by Doak et al. (2007): it amounts to 147 μM h, in remarkable agreement to the *in vivo* AUC at the threshold dose.

The good agreement between the *in vitro* and *in vivo* 'AUC—thresholds' is considered to further lend strong support to the threshold concept for EMS. The data provided by Lave et al. (2009a,b) also suggest that the half-life of EMS in longer in rats and monkeys than in mice. This implies that the dose to exceed the threshold for mutation induction, if measurable in rats and monkeys (and humans accordingly), would be lower than in the mouse. In the rat, the threshold for mutation induction may already be exceeded at ~10 mg/kg. This would be consistent with the observation that a dose of ~20–30 mg/kg/day EMS given in the drinking water for ~3 months does produce mammary tumours.

In summary, the maximal human exposure at 0.055 mg/kg is predicted to be $AUC_{hum0.055}$ = 13 μM h, this is about ~100-fold below the levels that are needed to produce a mutation increase in the mouse at 50 mg/kg and ~30-fold below the levels calculated in mice at the threshold dose (no observed effect level; NOEL) of 25 mg/kg ($AUC_{mouse\ 25}$ = 349 μM h).

Viracept patients ingesting the most contaminated batches of the medication have received doses of EMS which are 450-fold below the threshold (NOEL) dose for mutagenic effects in dedicated mouse genotoxicity studies. The maximal serum concentration of EMS in the patients is estimated to be 370-fold lower than the C_{max} at the threshold dose in mice. Even when considering the comparatively higher exposure levels of EMS in man, due to the slower clearance, the daily exposure of the patients to EMS was still about 30-fold below the exposure of mice at the threshold dose. The AUC based safety factor can be considered as presenting the lower boundary ('worst case'). The 'true' safety factor might approach the value of the C_{max} based calculation, but without detailed knowledge of the repair processes it is not possible to estimate how close the value will be approached.

It is emphasized that the threshold for mutation induction by EMS is similar in all investigated organs (GI-tract, bone marrow, liver). Dose fractionation of a single mutagenic dose into smaller doses abolished the mutagenic effect in all three organs (Gocke et al., 2009c). Hence, the calculated safety margins are considered to not significantly change with longer treatment durations than 28 days and would not be expected to differ between tissues.

This calculation of safety factors has been based on animal data in a non-disease model and extrapolations to humans were made based on a highly conservative cross species extrapolation of human C_{max} and AUC of EMS. We have also presented a review of the influence of factors such as a difference in susceptibility of HIV infected patients relative to the normal healthy population, co-medications, adult versus young organisms, species and organ differences in DNA repair activity and cell turnover. While such factors may be important for exposure to other types of agents, they are unlikely to affect the derived safety factors for the "EMS in Viracept" case.

We conclude that patients ingesting maximally contaminated Viracept tablets for the full 3 months period would not be expected to experience adverse genotoxic effects. Consequently, the risk for adverse tumourigenic or teratogenic effects highly likely does not exceed the background risk.

In deriving a general recommendation from our data for a "permitted daily intake" (PDE) calculation for EMS, we have nevertheless used safety factors to amount to a total of 10,000 (Müller and Gocke, 2009).

5. Overall summary on risk assessment for EMS in Viracept

As a result of an inappropriate cleaning process during production of the nelfinavir mesylate salt and incomplete product quality monitoring, Viracept tablets were contaminated with relatively high levels of EMS during February until April 2007. These tablets were taken by a fraction of Viracept patients who were potentially exposed to the contaminant EMS for a maximum duration of 3 months in spring 2007 (from first delivery of contaminated tablets to the pharmacies in March 2007 until the global recall end of May 2007) at levels of potentially up to 0.055 mg/kg/day.

In order to assess any risk for adverse consequences of this exposure we have initially performed a risk assessment based on the assumption of linear dose–effect relationships (Gocke et al., 2009b). In this initial assessment, we have compared the calculated risk with the only available reliable linear human risk assessment for a condition of exposure to a genotoxic carcinogen, i.e. the quite exact calculation of risk of cancer from diagnostic use of X-rays (Barrington de Gonzales and Darby, 2004). However, further to a series of toxicological and drug metabolism studies we now provide a solid basis for the conclusion that induction of mutations by EMS does not follow a linear dose–effect relationship as assumed for X-rays but follows a thresholded dose–response (Gocke et al., 2009c) with error-free repair of DNA alkylations below the threshold. Hence, it can be argued that the chance of adverse genotoxic effects at exposure levels below the threshold is practically zero. The same holds true for adverse effects (cancer, birth abnormalities) occurring in consequence of genotoxic events.

We are aware of the fact that the data argue for a clear difference in the dose–response for genotoxicity of EMS compared to other (alkylating) genotoxic agents, for which generally linear dose–effect relationships are assumed. In fact, the data for the other alkylating agent used in our experiments, ENU, clearly suggest linearity (or a threshold value considerably below 1 mg/kg/day) thus differentiating the two genotoxins so far until further data are generated at even lower dose levels for ENU. From the vast literature data that are available on genotoxicity of EMS, we are not aware of any publication that gives experimental evidence incompatible with the conclusions of a threshold as presented by our studies (Gocke et al., 2009a).

Based on our detailed exposure, distribution and metabolism studies in mice, rats and monkeys we have calculated that the patients exposed to EMS via ingestion of highly contaminated

Viracept tablets were exposed to levels of EMS clearly below the threshold exposure level for genetic damage. Consequently, none of them is expected to carry an elevated risk beyond her or his background risk for mutations and cancer. Further, the risk of birth defects for children born to pregnant women ingesting contaminated Viracept tablets during pregnancy as well as the risk of heritable defects in children born to parents who took contaminated Viracept tablets prior to conception is assessed as essentially zero.

Conflict of interest

Authors are employed at the company funding the research.

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